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(54) Title: METHOD OF SEQUENCING DOUBLE STRANDED DNA

(57) Abstract

The present invention provides a method of sequencing both strands of double stranded DNA wherein target DNA is subjected to at least one cycle of PCR using a pair of primers one of which is provided with a functional group for subsequent attachment to a first solid support or is already attached to said support and the other primer is provided with a different functional grouping permitting attachment to a second solid support, reacting the double stranded PCR products with said support or supports and separating the immobilised strands from each other and from the reaction solution, followed by sequencing of each of the said strands. Also provided is a kit for use in such a method.

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Method of sequencing double strand d DNA

This invention relates to a method of sequencing both strands of double stranded DNA.

There is an increasing demand for reliable sequencing of DNA, particularly in relation to the human genome project and the identification of DNA associated with pathological conditions. It is particularly desirable that such sequencing methods lend themselves to automation.

Recently, many technical improvements have been reported, although the major contributions concern the data evaluation, i.e. computer software. A filter method to prepare single stranded phage DNA has been described (Kristnesen et al (1987)), which may be developed into an automated procedure. Attempts to develop automated sequencing reactions by a centrifugal reagent handling device have also been described (Martin et al (1985)) as well as image processing programs for the detection of the bands on the radiograms (Elder et al (1985)). However, the most common approach has been to automate techniques with the aid of robots. Using such a strategy, systems for high-speed sequencing (Wada et al 1983)) and DNA template preparations (De Bonville et al 1987)) have been introduced.

A novel approach to automize the electrophoresis step has been described by several groups (Smith et al (1986), Ansorge et al (1987) and Prober et al (1987)) taking advantage of fluorescence instead of isotopes for labelling the DNA fragments. With these systems on-line detection can be achieved, which makes it possible to combine the three operations electrophoresis, detection and data handling into a single automated station. Such systems are therefore likely to be included in megabase sequencing strategies.

To obtain a completely automated sequencing protocol, it is therefore essential to also develop suitable automated methods for the first two operations (template preparation and sequencing reactions). For the latter operation a strategy involving solid phase techniques would facilitate automated handling of liquids in microliter quantities, which would be suitable for automated protocols.

Solid phase methods have proven to be very useful in molecular biology, in areas such as peptide synthesis, peptide sequencing and DNA synthesis. A large number of instruments are commercially available utilizing this technique. The advantage with a solid phase approach is usually a combination of good yields, reproducable reactions and easy automation.

At present there are, however, few reports on solid phase approaches to handling manipulatations of cloned DNA sequences for applications such as DNA sequencing reactions, site-directed manipulations or cDNA synthesis. DNA sequencing of oligonucleotides on anion-exchange supports (Rosenthal et al, 1985) has been described, but most attempts to automate DNA sequencing have been focused on the use of laboratory robots (Martin et al, 1985, and Wada et al, 1987).

WO 89/09282 describes a method of sequencing one strand of double stranded DNA whereby the latter is immobilised via one terminus of one of the two strands and subjected to strand separation prior to sequencing the immobilised strand.

The immobilized single stranded DNA for sequencing may also be produced by the polymerase chain reaction (PCR) technique whereby relatively small amounts of the DNA to be sequenced can be greatly amplified enzymically and according to a modification, also immobilized on a solid support. In the PCR technique, two oligonucleotide primers are selected which hybridise to respective sequences at or near the 5'-ends of the

coding and non-coding strands of the DNA to be amplified; after annealing to hybridise the primers to the target DNA, polymerisation is then effected using a suitable polymerase to produce a copy of each of the coding and non-coding strands incorporating the primers whereupon strand separation is effected, e.g. by conventional melting for example at 90°C. If an excess of the primer oligonucleotides is included in the medium as well as the four nucleotides required for synthesis, the separated new strands together with the original strands can serve as templates for a further cycle of annealing, polymerisation and strand separation. It will be seen that if this procedure is continued through a number of repeated cycles, the target DNA will be amplified exponentially while other DNA present will largely be unaffected. Recently, a thermophilic polymerase has become available, Taq 1, which can withstand the melting temperature needed for strand separation, thus avoiding the need to add polymerase at each repeat of the cycle as when using the Klenow polymerase used originally in PCR.

If one of the oligonucleotide primers is attached to a solid support such as a particle or, more preferably, carries means permitting attachment to a solid support such as biotin, the amplified DNA will be produced with means for immobilisation. Thus, the PCR technique can produce directly immobilized single stranded DNA ready for sequencing and may produce this directly from a bacterial colony by a method which is easy to automate and does not involve restriction cleavages and plasmid purification. A particular advantage of the use of a solid support in any of the reactions here concerned is the ease of separation from the reaction medium. Thus, in the PCR stage, the reaction medium can be readily removed by washing and a different polymerase introduced in an optimal buffer to begin the sequencing stage, e.g. a conventional

sequencing polymerase such as T7. Furthermore, the optimal concentrations of nucleotides and dideoxynucleotides can be maintained for sequencing by the Sanger method, independently of the concentrations used in the PCR step. The possibility of rigorous washing of the immobilized DNA provides more reproducible results in the sequencing stage. Furthermore, the so-called 'walking primer' technique is facilitated in Sanger sequencing whereby a primer can be used to sequence the first 500 base pairs of a long DNA molecule and after washing, the unchanged immobilized DNA is annealed to a primer initiating sequencing of the next 500 base pairs (using sequence information from the first stage), this procedure being continued until the whole DNA molecule has been sequenced.

In order to improve the reliability of the sequence information so obtained, it is possible to sequence the non-immobilised strand in the solution from which the immobilised strand has been removed. However, such a solution is normally at a high pH, due to the conventional use of sodium hydroxide in the strand separation stage, and has to be neutralised. Additionally, it is difficult to avoid diluting the solution containing the non-separated strand to a concentration optimal for sequencing.

We have found that it is possible to improve the sequencing of both strands of double stranded DNA by subjecting the target DNA to at least one cycle of a polymerase chain reaction (PCR) using a pair or primers each of which carries a different means of immobilisation so that the strands can be isolated separately for sequencing.

According to the present invention therefore there is provided a method of sequencing both strands of double stranded DNA wherein target DNA is subjected to at least one cycle of PCR using a pair of primers one of which is provided with a functional group for subsequent

attachment to a first solid support or is already attached to said support and the other primer is provided with a different functional grouping permitting attachment to a second solid support, reacting the double stranded PCR products with said support or supports and separating the immobilised strands from each other and from the reaction solution, followed by sequencing of each of the said strands.

In the preferred method of the invention, both primers preferably carry functional groups.

The target DNA may be from, for example, genomic or plasmid DNA or cDNA prepared in situ from target mRNA. The target DNA may be double stranded or single stranded since the PCR reaction will produce double stranded DNA from any single stranded target DNA. It will be appreciated that where the target DNA is single stranded, sequencing of a complementary strand will provide a check on the sequence information from the primary strand.

If the target DNA is present in very small quantities, it may be advantageous to subject the target DNA to several cycles of PCR before the immobilisation/separation procedure. Nested primers may also be used advantageously, that is, a first primer pair may be used to amplify the target DNA initially and a second primer pair may then be introduced for the final PCR cycles in order to provide additional selectivity in amplifying the correct target DNA. The PCR primers used in accordance with the invention may, in that case, only be used in the final PCR stage. Nested primer PCR techniques are described in the literature (see for example Wahlberg et al., P.N.A.S. 1990, 87: 6569-73).

The target DNA may vary considerably in length, from a few base pairs to up to for example 30 to 40 thousand base pairs depending on the application.

The functional groups could be any groupings which can be incorporated into deoxynucleotides and which have a strong interaction to a substance which can be immobilized on a solid-support. Examples of such groups include biotin - avidin, biotin - streptavidin, and cysteine - thiol groups, or any suitable hapten-anti-hapten binding pair. One example of a suitable functional group is thus biotin. Suitable haptens in this regard include digoxigenin, dinitrophenol (DNP), 3-nitro-4-hydroxy-5-iodophenylacetic acid (NIP), 3-nitro-4-hydroxy phenylacetic acid (NP) or modified nucleotides. The anti-hapten may be a complete antibody, or an antibody fragment retaining binding activity e.g. $F(ab)_2$,Fab or Fv fragments. Thus for example the first of a pair of primers according to the invention may carry a biotin functional group, and the second a hapten such as digoxigenin, e.g. at the 5' end, and may be immobilised on first and second solid supports carrying avidin or streptavidin, and the anti-hapten (e.g. anti-digoxigenin) respectively. Methods for attaching such functional groups to the primers, and their corresponding binding partner to the solid supports are well known in the art.

Any conventional solid support material, such as Sepharose (Pharmacia, Sweden), magnetic beads, filters or capillaries, to which the substance can be sufficiently coupled for the present purposes, may be used. Magnetic beads are particularly preferred since they permit ready and simple separation of the immobilised DNA strands from solution. The methods for coupling or immobilizing a functional group to such carrier material are well-known and need not be described in any detail herein. It is also possible to use surfaces of microtiter wells as a means for coating. Strand separation may be effected by conventional methods, such as by the use of alkali (e.g. 0.15M NaoH) or by heat treatment.

Sequencing may be effected by the Maxam Gilbert or, more preferably, primer extension methods such as the Sanger dideoxy method. Any of the conventional variations of these methods may be used but it is preferred to use the Sanger method with separate tubes for the A C G and T reactions with labelled primers or labelled nucleotides (e.g. with means enabling chemiluminescent or radioactive detection). Alternatively the A C G and T reactions may be performed in a single tube, each dideoxy equivalent being differently labelled.

Solid-phase sequencing on an immobilised template is generally preferred according to the invention, although other methods may of course be used. In this case the DNA chains to be sequenced may be liberated from the inert support by, for example, treatment with formamide.

According to one preferred method of the invention, following the PCR step using two differentially-functionalised primers, the mixture is contacted with said first solid support carrying a substance with affinity for the functional group carried by the first primer leading to directed immobilisation of DNA fragments carrying the functional group. Single stranded DNA is obtained by melting the strands, either by alkali or heat treatment and the non-immobilised strand is eluted. The first solid support carrying the immobilised strand is separated from the reaction mixture and the eluted strand is subsequently contacted, following if necessary appropriate washing and neutralisation steps, with the second solid support which carries a substance with affinity for the functional group provided on the second primer.

For Sanger-type sequencing, eg. standard sequencing or cycle sequencing, general forward or reverse sequencing primers may then be annealed to the two resulting immobilised single stranded templates and

sequencing reactions performed under standard conditions. The extended oligonucleotides can be labelled using different strategies, eg. radio-isotopes, chemiluminesence or fluoresence which may be incorporated either during the extension or as a labelled primer. The newly synthesized labelled oligonucleotides are eluted by another melting step leaving the template available for the next sequencing reaction. The annealing and extension is repeated to obtain specific fragments for all four nucleotides and the four samples are loaded onto sequencing gels.

The various reactants in the method of the invention may conveniently be supplied in kit form. Such kits form a further aspect of the invention.

Thus in a further aspect the invention provides a kit for the sequencing of both strands of double stranded DNA comprising

(a) a pair of PCR primers, the first of which is provided with a functional group for subsequent attachment to a first solid support, preferably magnetic particles, or is already attached to said support; and the second of which is provided with a different functional grouping permitting attachment to a second solid support;

(b) either first and second solid supports, preferably magnetic particles, carrying groups binding with the functional groups provided on said first and second primers; or said second support only;

(c) one or more polymerases; and one or more of:

(d) appropriate buffers;

(e) dideoxynucleotides ddT, ddA, ddC and ddG;

(f) dideoxynucleotides or an oligonucleotide, each carrying, or adapted to carry a label.

The primers may be specific for the target DNA or may be standard PCR primers.

By permitting sequencing of both strands of the double stranded DNA under identical conditions, it is possible to obtain improved accuracy in sequencing which is of value in the identification of unknown DNA molecules, for example in diagnosis or basic research.

The solid phase approach of the present invention is suitable for both manual and automated sequencing and may be applied to plasmid DNA obtained directly from bacterial colonies through the PCR. It thus provides an integrated method for template purification, strand separation and sequencing of both strands of the DNA template; amplified double-stranded DNA, "labelled" on each strand through the functional groups carried on the primers may, without purification, be selectively captured and immobilised on a suitable support, preferably magnetic beads (e.g. Dynabeads of Dynal AS), followed by strand separation, immobilisation of the second strand and simultaneous sequencing of the two separated immobilised strands. The solid-phase approach ensures that the amplification and sequencing reactions can be performed under optimal conditions.

The invention will now be described by means of the following non-limiting Example.

<u>Example 1</u>

Solid phase sequencing of both strands of an abrin gene clone using Dynabeads M280-streptavidin and Dynabeads coated with anti-digoxigenin as solid supports.

<u>Materials</u>:

(a) <u>Plasmid clone : pGA7.3</u>

(G Evensen et al, Journal of Biological Chemistry

266 6048-6052,1991)
comprising an abrin A gene inserted in vector pGEM 7Z f (+) (Promega)

(b) Solid supports

(1) Dynabeads M280-Streptavidin (Dynal A.S. Oslo, Norway)

(2) Dynabeads coated with anti-digoxigenin.

(c) Anti-digoxigenin (Catalogue No. 1333062 Boeringer Mannheim)

(d) Primers used in the PCR amplification

5'digoxigenin - GCTTCCGGCTCGTATGTTGTGTG 3'
5'biotin - AAAGGGGGATGTGCTGCAAGGCG 3'

The primers are identical to regions within the lac Z gene which common cloning vectors such as pGEM 7Z f (+) contain.

(e) Buffers

2 x B & W	=	10mM Tris HCl (pH 7.5) 1mM EDTA 2.0M NaCl
20 X PBS	=	$NaH_2PO_4.H_2O$ 3.12g $NaHPO_4.2H_2O$ 19.6g NaCl 0.15M 162g dH_2O to 1l adjust pH with NaOH to 7.4 1 x stock is working solution

(f) DNA Sequencing Kit

Sequenase version 2.0 DNA sequencing kit from USB (United States Biochemical Corporation)

Methods

1. Preparation of Dynabeads M-280 Streptavdin

20μl (200μg) of the Dynabeads were washed once in 20μl of 2 x B & W buffer using a Magnet (Dynal MPC-E) and an Eppendorf tube. After washing, the beads were resuspended in 40μl 2 x B & W buffer.

2. Preparation of anti-digoxigenin coated Sheep-anti-Mouse IgG Dynabeads M-280

Concentration of antibodies is 3.4 μg per mg beads. Coating buffer is PBS pH 7.4 (0.0075M)

Protocol:

1. The sheep anti-mouse beads were washed once in PBS buffer.
2. 3.4 μg antibodies/mg beads were used at a concentration of 100 mg beads/ml PBS buffer. The solution was allowed to rotate at a 4°C.
3. The coated beads were washed twice with PBS-buffer and stored in PBS-buffer containing 0.1 % BSA, 0.02% disodium azide.

3. PCR Conditions

(i) 5 pmol of each labelled primer (carrying either biotin or digoxigenin)

(ii) vector pGA7.3

(iii) The Perkin Elmer Cetus Ampli taq kit was used

(iv) PCR cycles 96°C 0.5 minute
65°C 1.0 minute

72°C 2.0 minute

(at a volume of 50µl)

(v) A Hybaid thermo cycler was used.

4. Immobilization of the PCR product

40µl of the amplified PCR product was added to the 40µl of prewashed beads (step 1) and incubated at room temperature for 15 minutes keeping the beads suspended.

5. Melting the DNA duplex

The beads carrying the immobilized PCR product were washed once with 40µl 2 x B & W.

After the supernatant was removed, 8µl of 0.1M NaOH was added and incubated at room temperature for 10 minutes.

6. Separating the DNA strand

Using the MPC-E the supernatant containing the eluted strand was transferred to a new Eppendorf tube and a new treatment of 8µl 0.1 M NaOH was performed.

The Dynabeads with the immobilized biotinylated strand were washed once with 50µl 0.1M NaOH, once with 40µl B & W, and once with 50µl TE buffer. The supernatant was removed and the volume was adjusted with distilled water according to the template volume in the sequencing protocol.

7. <u>Immobilizing the digoxigenin labelled strand</u>

The saved supernatant 2 x 8μl (step 6) was neutralized with an equal volume of 0.1M HCl and 2μl of 1M tris-HCl pH7.4. The volume was adjusted to 50μl using PBS pH7.4.

500μg Dynabeads anti-digoxigenin (step 2) were washed once in PBS and resuspended in 50μl PBS and added to the neutralized digoxigenin labelled strand solution. The mixture was incubated at room temperature for 30 minutes with occasional mixing after washing once with 100μl PBS and 50μl 1 x TE buffer the beads were resuspended in water according to the template in the sequencing reaction.

8. <u>Sequencing reaction</u>

The manufacturer's recommended protocol was followed (USB).

(a) annealing mixture:

I) 7μl of the biotin labelled strand immobilised on Dynabeads M280 Streptavidin (200μg) was added to <u>1μl reverse sequencing</u> primer and 2μl sequencing buffer. Annealing was carried out by heating for 2 minute at 65°C then cooling slowly to < 35°C.

II) 7μl of Dynabeads anti-digoxigenin carrying immobilized digoxigenin labelled primer was added to 1μl forward sequencing primer and 2μl sequencing buffer.
Annealing was by heating for 2 minutes at 65°C then cooling slowly to <35°C.

The rest of the protocol followed the manufacturer's instructions.

After completing the reaction the samples were heated to 75°C for 2 minutes immediately before loading. After magnetic separation 4μl of each reaction was loaded on to a sequencing gel.

The procedure of Example 1 is illustrated schematically in Figure 1.

CLAIMS

1. A method of sequencing both strands of double stranded DNA wherein target DNA is subjected to at least one cycle of PCR using a pair of primers one of which is provided with a functional group for subsequent attachment to a first solid support or is already attached to said support and the other primer is provided with a different functional grouping permitting attachment to a second solid support, reacting the double stranded PCR products with said support or supports and separating the immobilised strands from each other and from the reaction solution, followed by sequencing of each of the said strands.

2. A method as claimed in claim 1 wherein each of said pair of primers is provided with a functional group for attachment to a solid support, said functional groups being different from each other.

3. A method as claimed in claim 1 or claim 2 wherein the PCR step is performed using nested primers.

4. A method as claimed in claim 3 wherein only the inner pair of nested primers are provided with said functional groups.

5. A method as claimed in any one of claims 1 to 4 wherein the functional group is a biotin or thiol group or a hapten, and said solid supports carry groupings binding thereto.

6. A method as claimed in claim 5 wherein one of said pair of primers carries a biotin functional group and the other carries digoxigenin.

7. A method as claimed in claim 5 wherein the solid support is in particulate form or is a microtitre well.

8. A method as claimed in claim 5 wherein the solid support is in the form of magnetic beads.

9. A method as claimed in any one of claims 1 to 8 wherein sequencing is effected by a primer extension method.

10. A method as claimed in any one of claims 1 to 9 wherein sequencing is effected by the Sanger method.

11. A kit for the sequencing of both strands of double stranded DNA comprising

(a) a pair of PCR primers, the first of which is provided with a functional group for subsequent attachment to a first solid support, or is already attached to said support; and the second of which is provided with a different functional grouping permitting attachment to a second solid support;

(b) either first and second solid supports carrying groups binding with the functional groups provided on said first and second primers; or said second support only;

(c) one or more polymerases; and one or more of:

(d) appropriate buffers;

(e) dideoxynucleotides ddT, ddA, ddC and ddG;

(f) dideoxynucleotides or an oligonucleotide, each carrying, or adapted to carry a label.

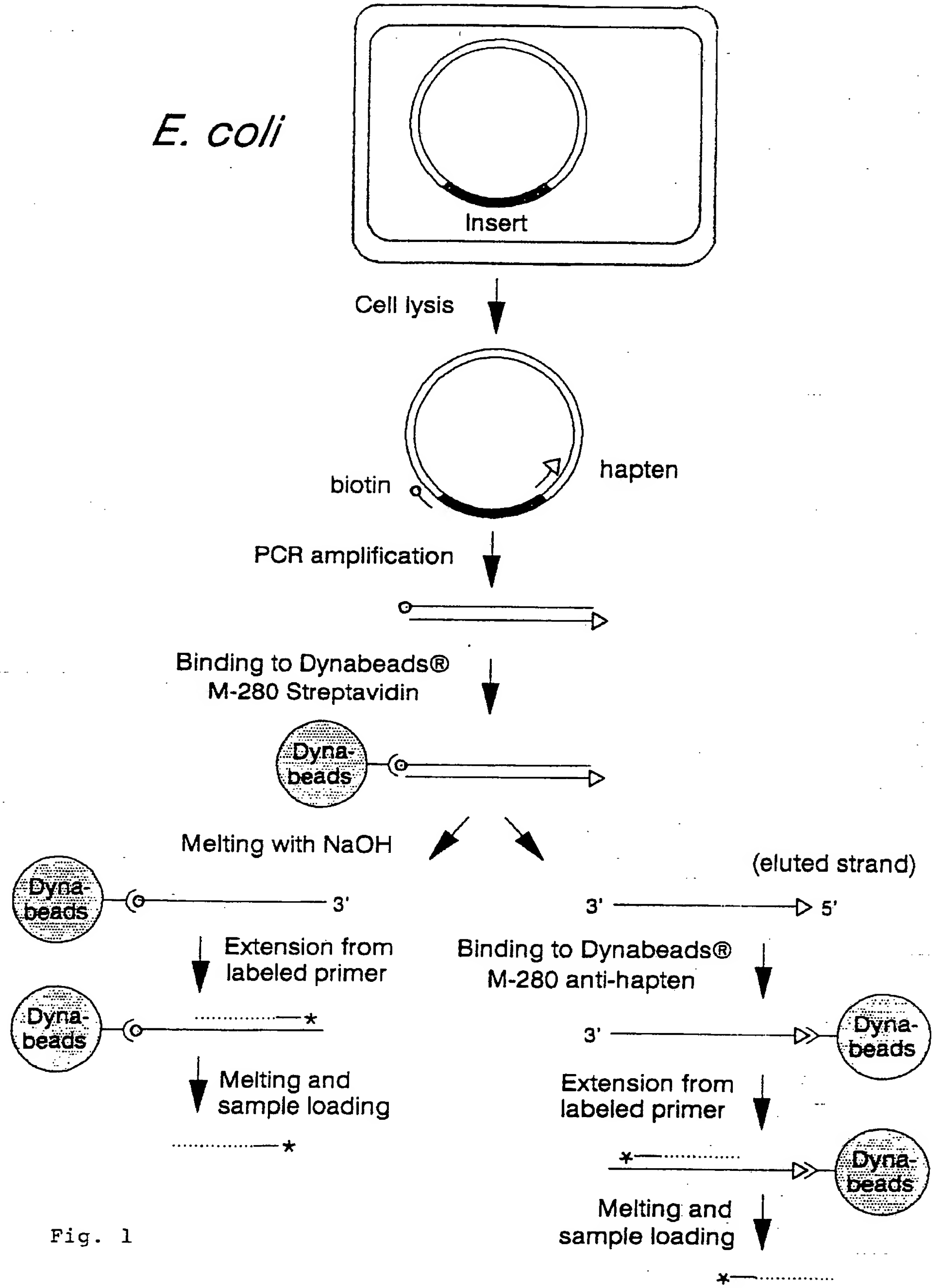
E. coli
Insert
Cell lysis
biotin
hapten
PCR amplification
Binding to Dynabeads®
M-280 Streptavidin
Dyna-
beads
Melting with NaOH
(eluted strand)
Dyna-
beads
3'
3'
5'
Extension from
labeled primer
Binding to Dynabeads®
M-280 anti-hapten
Dyna-
beads
3'
Dyna-
beads
Melting and
sample loading
Extension from
labeled primer
Dyna-
beads
Melting and
sample loading

Fig. 1

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 92/02421

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/68

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	EP,A,0 371 437 (ORION-YHTYMÄ OY) 6 June 1990 see page 2, line 30 - page 6, line 40; claims 6,7,9	1,2
A	DE,A,3 816 934 (RAMALHO-ORTIGAO ET AL.) 23 November 1989 see page 4, line 27 - line 64; figure 2	1,2,5
A	EP,A,0 437 774 (BOEHRINGER-MANNHEIM GMBH) 24 July 1991 see the whole document	1,6
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 86, April 1989, WASHINGTON US pages 2423 - 2427 D.J. KEMP ET AL. see abstract	1,3

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IV. CERTIFICATION

2

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11 FEBRUARY 1993	03.03.93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	LUZZATTO E.R.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

EP 9202421
SA 65880

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0371437	06-06-90	AU-B- 623200	07-05-92
		AU-A- 4574989	07-06-90
		CA-A- 2004056	29-05-90
		JP-A- 2219600	03-09-90
DE-A-3816934	23-11-89	EP-A- 0417161	20-03-91
		WO-A- 9001562	22-02-90
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		JP-A- 4211400	03-08-92

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